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Telomerase, the enzyme which maintains the repetitive DNA sequences found at the ends of chromosomes, is an excellent candidate for an anti-tumor drug target. Almost all cancer cells have and need telomerase, while normal somatic cell neither have nor need telomerase. Human telomerase contains at least three components: a catalytic reverse transcriptase called hTRT; an RNA molecule called hTR that provides the template for the reverse transcriptase, and a protein of undefined function called TP1 or TLP1. We are attempting to find additional components of human telomerase. We have used a three-hybrid screen to find additional molecules that bind to the telomerase RNA. We have found some promising candidate binders, including (1) DEK, (2) poly (ADP-ribose) polymerase, (3) the human Rad21 homolog, and (4) c-myc. We are now assessing the *in vivo* relevance of these proteins to telomerase function. We have shown that *in vitro*, telomerase activity can be reconstituted with just two components, hTRT and hTR.

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Bruce Tuteur Jan 25, 1998

PI - Signature Date

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Introduction.

Chromosome ends are capped by structures called telomeres. Since DNA polymerases synthesis DNA only in the 5' to 3' direction, they cannot completely replicate the ends of a linear molecule (Watson 1972; Olovnikov 1973). The problem is solved by an enzyme called "telomerase", which adds repetitive DNA (TTAGGG, in mammals) to chromosome ends to balance the loss of sequences at replication (Greider and Blackburn 1985; Greider and Blackburn 1987). Germ line cells have telomerase, and this is how telomeres are maintained. However, normal somatic cells lack telomerase activity, and so the telomeres of somatic cells get shorter and shorter with every cell doubling (Harley et al. 1990). After about 80 doublings, telomeres are very short or absent, and the cells are unable to double further. Somatic cells have a limited replicative capacity (Hayflick 1961), and the lack of telomerase seems to be the reason for this, since expression of telomerase in otherwise normal fibroblasts allows them to double indefinitely, escaping the Hayflick limit (Bodnar et al. 1998).

Immortal cells must have a method of maintaining telomeres, and indeed it has been found that immortalized cell lines and all or almost all tumor cells have telomerase (e.g., Kim et al. 1994; Langford et al. 1995; Hiyama et al. 1996; Sommerfeld et al. 1996). Thus, it seems that switching on telomerase is an essential step in tumorigenesis. Telomerase may be the perfect target for anti-tumor drugs--normal somatic cells neither have it nor need it, while tumor cells do have it and do need it. The long term objective of this proposal is to enable the development of anti-telomerase drugs for cancer therapy.

However, telomerase is a non-abundant and poorly characterized enzyme. It has at least three components: A catalytic reverse transcriptase subunit called hTRT (human Telomerase Reverse Transcriptase) (Nakamura et al. 1997; Meyerson et al. 1997; Harrington et al. 1997); an RNA molecule called hTR (human Telomerase RNA) that provides the CCCTAA template for the reverse transcriptase (Feng et al. 1995); and a possible RNA-binding protein called TP1 or TLP1 of poorly defined function (Harrington et al. 1997; Nakayama et al. 1997). The amounts of telomerase that would be needed for drug screening are not available. The objective of this proposal is to further define the components of human telomerase and their roles, so that large amounts of telomerase can be produced for drug screening and other studies.

Some of the original proposal, including all of Aim 2, was directed towards cloning the catalytic subunit hTRT, which had not been done at that time. However, hTRT has now been cloned by Cech and others (Nakamura et al. 1997; Meyerson et al. 1997). Therefore we are not pursuing Aim 2, but instead will undertake a deeper characterization of the telomerase components already in hand. In addition, we will reconstitute telomerase on a large scale potentially suitable for drug screening.

Body of the Report.

The main goal of the proposal was to isolate protein components of human telomerase. However, shortly after our work on the proposal began, the key, catalytic component of human telomerase, hTRT, was cloned by other groups. Cech and co-workers had recently purified, identified, and cloned the telomerase reverse transcriptase from Euplotes (Lingner et al. 1997), and the human homolog appeared in the EST database as a consequence of cDNA sequencing projects (Nakamura et al. 1997; Meyerson et al. 1997; Harrington et al. 1997). Furthermore, a second protein component called TR1/TLP1 was found, partly by purification, and partly by homology to a Tetrahymena telomerase component (Harrington et al. 1997; Nakayama et al. 1997). Thus, by the first few months of our project, a total of three human telomerase components were known: the telomerase RNA, hTR; the catalytic reverse transcriptase, hTRT; and the Tetrahymena p80 homologue of unknown function, TR1/TLP1. The fact that these components had been found made us alter our aims and methods. There was no reason to think that human telomerase would have only three components, and so we continued to search for other proteins that might be components of the complex, or at least be associated with the complex. To some extent we were able to use the methods originally described, particularly the three-hybrid screen. We decided not to proceed with the original Aim 2, functional cloning, because this was directed entirely at cloning the catalytic reverse transcriptase, which had already been found.

Aim I. The three-hybrid screen with hTR.

Aim 1 was to do a three-hybrid screen with hTR. The three hybrid screen (Fig. 2) (Sengupta et al. 1996) is an adaptation of the two-hybrid screen. It is designed to find RNA-binding proteins. It relies on the fact that the coat protein from bacteriophage MS2 binds strongly and specifically to a region of the MS2 genomic RNA. The first hybrid is a lexA-MS2 coat protein fusion. The second hybrid is the relevant portion of MS2 genomic RNA fused to the RNA of interest (in this case, hTR). The third hybrid is the library hybrid; that is, random cDNAs fused to the Gal4 activating domain. The first two hybrids are expressed constitutively in a screening yeast strain that has lexA binding sites upstream of two reporter genes, *HIS3* and B-galactosidase. The strain is then transformed with a library. When a library plasmid has an hTR-binding protein fused to the Gal4 activating domain, this will bind to the hTR-MS2 fusion, which is bound to the MS2 coat protein-lexA fusion. Thus, the RNA fusion acts as a bridge to bring the lexA DNA binding domain together with the Gal4 activating domain, turning on the reporter genes. Dr. Fields' lab has demonstrated that the screen works in several test cases, for instance the interaction between the iron response element (IRE, RNA) and iron response protein (IRP) (Klausner et al. 1993).

The weakness of the three-hybrid screen is that it tends to pick up any kind of RNA binding protein. To partly prevent this, we made a fusion between the IRE (Iron response element) RNA and MS2, and used this as a negative control; that is, we demanded that interacting clones from the library interacted with hTR-MS2 RNA, but not with IRE-MS2 RNA.

We put the hTR-MS2 RNA fusion (marked with *URA3*) and the IRE-MS2 fusion (marked with *ADE2*) into the same yeast strain, and screened with a cDNA-*GAL4*

(activating domain) library (marked with *LEU2*) from immortal human cells. Transformants were selected on -leu -his -ade 10 mM 3-aminotriazole (3-AT) plates. (3-AT inhibits the His3 enzyme, the reporter gene for the screen, and 10 mM 3-AT demands fairly strong activation of *HIS3* expression.) In multiple experiments, we were able to screen about 2 x 10^8 yeast transformants; the original library had a complexity of about 1 x 10^8. We obtained a total of just under 1,000 primary transformants able to grow on 10 mM 3-AT. Each of these were patched onto –ade 5-FOA plates to remove the hTR-MS2 fusion, but leave the IRE-MS2 fusion in place. After this step, we found there were about 76 clones where loss of the hTR-MS2 fusion now prevented the clones from growing well on –his 3-AT plates, suggesting that these 76 clones had a specific interaction with hTR. These 76 clones are our best candidates for telomerase components, since they require the telomerase RNA (and not the irrelevant IRE-MS2 RNA) for high-level expression of *HIS3*.

Plasmid DNA was recovered from all 76 clones. Restriction mapping was done to identify duplicates, and a portion of representative clones was sequenced. The sequence was matched against the EST and other databases to identify the clones. The clones obtained apparently represent 36 different proteins, and are summarized below:

Clones with no match to database (9 clones): 1a, 231, 313, 319, 321, 329, 343, 397, 459.

Genes in EST or other databases with match to one or more clones:

1. Hax-1 (13 clones). Poorly understood.
2. nuclear factor NF90 homolog. (5 clones). RNA binding protein. Poorly understood.
3. FRG1. Poorly understood.
4. **DEK. Weak homology to Tetrahymena p95 telomerase component.** Proto-oncogene. Poorly understood.
5. HMG1 (2 clones). High mobility group protein.
6. Inosine monophosphate dehydrogenase.
7. Proteosome subunit HC3 (5 clones)
8. Saposin protein A-D. Poorly understood.
9. Hsp90
10. Epstein-Barr virus mRNA (2 clones)
11. ribosomal protein S6 (2 clones)
12. nuclear factor NF 45 homolog
13. NAD(H)-specific iso-citrate dehydrogenase (3 clones)
14. I-plastin mRNA
15. Erp31. Poorly understood.
16. activin beta-A subunit
17. **poly (ADP-ribose) polymerase**
18. **c-myc**
19. K1AA0098
20. **K1AA0078, Rad21 homolog**
21. K1AA0026
22. BAC397c4
23. Thymidine kinase

24. FBKIII 11c tyrosine kinase
25. Human cyclin C, 15 clones
26. Proteasome subunit RC6, 2 clones
27. Beta-hemolysin

These results are neither highly encouraging nor highly discouraging. On the discouraging side, many of the genes seem irrelevant to telomeric processes. However, any screen of this type gets many irrelevant genes. Also discouraging was the fact that we failed to get TR1/TLP1, a protein known to bind hTR. However, TR1/TLP1 is a rare and enormous protein, and the hTR binding region is near the N-terminus (Harrington et al. 1997), so it is unlikely to be represented in the three-hybrid cDNA library. On the encouraging side, we did not get large numbers of abundant but irrelevant RNA-binding proteins (for instance, we might have gotten dozens or hundreds of clones of ribosomal proteins, or splicing proteins, or hnRNP proteins). This implies that we did achieve some specificity. Also, some of the clones obtained are somewhat relevant to telomere metabolism.

The four clones of the most potential interest are (1) DEK; (2) poly (ADP-ribose) polymerase; (3) KIAA0078, the Rad21 homolog; and (4) c-myc. **DEK** is a very poorly understood gene, but it has been noted to be a potential oncogene (Dong et al. 1998), and it has some slight homology to Tetrahymena p95, a *bona fide* telomerase component (though of unknown function) (Collins et al. 1995). **Poly (ADP-ribose) polymerase** (PARP) is involved in DNA repair, and a homolog of poly (ADP-ribose) polymerase called tankyrase was recently found as a two-hybrid positive with the telomere-binding protein TRF1 (Smith et al. 1998). Furthermore, poly (ADP-ribose) polymerase shares a domain with BRCA-1, another protein potentially involved in DNA repair and in cancer (A. Neuwald, pers comm). DNA repair proteins may be relevant to telomere metabolism; a telomeric end is in some ways like a double strand break, and in the yeast *S. cerevisiae*, some repair proteins involved with double-strand break repair are also involved in telomere maintenance. **KIAA0078** (hsRad21) is the human homolog of the DNA repair gene *rad21* of *Schizosaccharomyces pombe* (Birkenbihl and Subramani, 1992), and of the DNA repair gene *RHC21* of *S. cerevisiae*. These are double-strand break repair genes, and so, like poly (ADP-ribose) polymerase, they are potentially involved in telomere metabolism. Finally, **myc** is a known oncogene, and telomerase is under the transcriptional control of myc (Wang et al. 1998). Conceivably there is a feedback loop between myc and the telomerase RNA that affects the transcription of telomerase components.

Future Experiments with Aim I.

A. Are the associations of DEK, PARP, hsRad21 and c-myc with hTR real? To see whether the apparent associations between hTR and our four most promising candidates actually occur *in vivo*, we will do co-immunoprecipiation experiments. Previously, we showed that the Est1 protein of yeast binds to the yeast telomerase RNA *in vivo* (Steiner et al. 1996). This was done by immunoprecipitating Est1, and using Northern blotting to show that the yeast telomerase RNA was specifically in the immunoprecipitates. We will do these same kinds of experiments with DEK, PARP, hsRad21, and c-myc. Antibodies to DEK, PARP and c-myc are already available. We

will clone hsRad21 into a mammalian expression vector, and tag it with a triple HA. We will then immunoprecipitate the four proteins in cells expressing hTR, and see if hTR co-precipitates. Of course, we will also look for irrelevant RNAs as a negative control, to see if any co-precipitation is specific. If hTR does specifically co-precipitate, it will be an encouraging sign that there are real *in vivo* interactions between hTR and the proteins of interest.

B. Analysis of novel and poorly understood genes. There were 9 clones which did not match any sequence in the database. As the database grows, we will continue to check for matches with these 9 clones. In addition, we plan to use these 9 clones as probes in Northern analysis. RNAs from different primary cells or transformed cell lines will be tested, and we will see if the expression pattern of any of the 9 clones correlates with the presence of telomerase activity in the cell strains or cell lines. We will also probe these Northerns with at least some of the clones for poorly understood genes (e.g. Hax-1, FRG1, NF90, NF45, KIAA0098, KIAA0026, BAC397c4).

Aim II. Functional Cloning of the Catalytic Subunit.

This aim was not pursued, because the catalytic subunit was cloned by Cech and co-workers.

III. Other Progress: Reconstitution.

Yeast telomerase has at least four components: Est1 (an RNA-binding protein), Est2 (the catalytic subunit), Est3 (function unknown), and Tlc1 (the telomerase RNA). It is not yet clear how many of these are needed for activity in vitro, though it is likely that both Est2 and Tlc1 are. So far, human telomerase has three subunits: hTR, hTRT, and TR1/TLP1. To see which of these are needed for activity in vitro, we made hTR by *in vitro* transcription, and hTRT by *in vitro* transciption/translation. The mixture of these two components had telomerase activity as assayed by the TRAP assay. Similar results have been obtained by other labs (Weinrich et al. 1997; Beattie et al. 1998). Thus, these seem to be the only two essential components for telomerase activity (unless the reticulocyte lysate used for translation added some important components). However, we cannot say whether this minimal complex is as efficient as the natural complex, nor whether it would successfully recognize a telomere in vivo, nor whether it would be properly regulated.

IV. Future Plans.

Future plans with the candidates from the three-hybrid screen have been described above.

In addition, now that hTRT has been cloned, we will look for further components of human telomerase by doing a yeast two-hybrid screen with hTRT. We have all necessary libraries, strains, and other reagents for this screen.

Finally, since we have found that telomerase activity can be reconstituted from just hTR and hTRT, we hope to make large quantities of human telomerase via recombinant DNA. Because the lab has expertise in yeast, and because other labs are undoubtedly trying to achieve the same ends using hTRT expressed in bacteria or insect cells, we will try expressing hTRT in the yeast *Pichia pastoris*. Telomerase reconstituted

in this way could be useful for biochemical studies of the enzyme, and also for drug screens.

Conclusions.

The initial stages of Aim I have been completed, and results are moderately encouraging. We have at least four reasonable candidates for genes that may interact with the telomerase RNA, and we are further investigating these genes. In addition, we are looking for novel components of telomerase using a two-hybrid screen with hTRT; we have reconstituted telomerase activity on a small scale, and we are planning to reconstitute activity on a large scale.

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Appendicies

None